Journal of Surgical Oncology 2000;73:95–99

Expression of Matrix Metalloproteinases and the Tissue Inhibitors of Metalloproteinases and Their Local Invasiveness and Metastasis in Chinese Human Pancreatic Cancer

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Background and Objectives: The objective was to evaluate the potent role of matrix metalloproteinases(MMPs) and the tissue inhibitors of metalloproteinases(TIMPs) in processes leading to metastasis and local invasiveness of Chinese human ductal adenocarcinomas of the pancreas. We also evaluated a possible biological association between the gene expression and clinical manifestations.
Methods: Northern blot and in situ hybridization have shown MMP and TIMP gene expression in the pancreas and alterations associated with neoplastic transformation. Fifteen cases of surgical pancreatic specimens were examined, using cDNA probes to MMP2, MMP9, and TIMP1. Findings were correlated with the size of tumor section, CA19-9, pathological classification, thrombosis, and infiltration of capsule and lymphonoids.
Results: Increased levels of the mRNA of MMP2, MMP9, and TIMP1 genes, MMP2≈MMP9<TIMP1, were found in pancreatic cancer tissues examined. Low levels of transcripts for MMP2, MMP9, and TIMP1 were detectable in pancreata of organ donors. Transcripts coding for MMP2, MMP9, and TIMP1 were found in both stroma and tumor cells. However, gene expression of MMP2, MMP9, and TIMP1 has shown an obvious correlation with the infiltration of capsule cells, surrounding lymphonoids and specific histopathological features.
Conclusions: We concluded that the imbalance between MMPs and TIMPs may help physicians to assess the metastatic potential and then tell the prognosis of individual patients.
J. Surg. Oncol. 2000;73:95–99.

KEY WORDS: pancreatic adenocarcinoma; pathology; prognosis

INTRODUCTION

In China, the incidence of pancreatic cancer seems to be increasing. Pancreatic ductal adenocarcinomas show a dense connective tissue reaction [1]. A strong desmoplastic reaction of extracellular matrix (ECM) proteins is involved in the local invasiveness and metastasis in pancreatic cancer [2]. Matrix metalloproteinases (MMPs), which degrade the various ECM components, are believed to play key roles in these processes. MMP activity is strictly controlled at gene expression, proenzyme activation, and inhibition by tissue inhibitors of metalloproteinases (TIMPs) [3]. It is of significance to show that overexpression of MMP genes and ECM desmoplastic

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Accepted 1 October 1999

reactions are associated with the invasive and metastatic pancreatic ductal adenocarcinomas. In this study, we analyze the balance between the gene expression of MMPs and their specific inhibitors of metalloproteinases in the pancreas and alterations associated with neoplastic transformation. Relations between the gene expression and the infiltration of surrounding lymphonoids and specific histopathological features were studied.

MATERIALS AND METHODS

Tumor Cell Lines and Tissue Samples

Cell lines from human pancreatic ductal adenocarcinomas (PaTu8902 and PaTu8988) were donated by H.P. Elsasser(Phillips University, Marburg, Germany). Pancreatic cancer tissues were obtained from 15 patients (6 females, 9 males) undergoing pancreatic surgery. Normal pancreatic tissues were obtained from 8 males through an organ donor program. All tissue samples were either frozen in liquid nitrogen (samples for Northern blot) immediately after surgical removal or fixed overnight in 10% neutral-buffered formalin and embedded in paraffin (samples for in situ hybridization [ISH]). Surgical pancreatic specimens were classified as ductal carcinoma (12 cases), acinar carcinoma (1 case), and others (2 cases). Among 15 cases of pancreatic cancer, there were 10 in the head, 4 in the body and tail, and 1 in the whole pancreatic gland.

Probes

All cDNA probes were obtained as plasmids and transformed in competent bacteria (*Escherichia coli*, XL1-blue). cDNA probes for MMP2, MMP9, and TIMP1 were a kind gift of Dr. Zh.K.Wang (Cardiothoracic Surgery Institute, Changhai Hospital, Shanghai, China). Probes were random primed-labeled with [^{32}P]dATP, yielding specific activities in the range of 2.38–4.76 × 10^4Bq/μm.

Northern Blot Analysis

Total RNA was separated on 1% agarose gels containing 50% formamide and transferred to Hybond N membranes (Huamei, Shanghai, China). Cloned DNA probes labeled by random hexamer priming using [^{32}P]dATP as a radioactive label were hybridized at 42°C in a solution containing 50 m*M* Tris-HCl, pH7.5, 1 m*M* NaCl, 50% formamide, 10 × Denhardt's solution, 10% dextran sulfate, 1% SDS , 0.1% sodium pyrophosphate, and salmon sperm DNA (100 μg/ml) in 0.1 ml/cm^2 of membrane. Hybridization was performed for 12–15 h (2.38–4.76 × 10^6 Bq/ml). Membranes were washed in 2 × SSC (1 × SSC, 150 m*M* NaCl and 15 m*M* sodium citrate), 0.5% SDS at 65°C and then at 37°C in 0.1 × SSC and exposed to Kodak XAR-5 film at −70°C using an intensifying screen. Evaluation of hybridization signals was semiquantificational in relation to the background signal and the signal obtained on the same Northern blot with each cDNA probe, using a VIDAS image analyzer (SX-100, Fudan University, Shanghai, China). The gray degree value of signals obtained was then converted into a caculable parameter and the gene expression rate was valued in accordance with the formula: (MMPs or TIMP1/β-actin) × 100%.

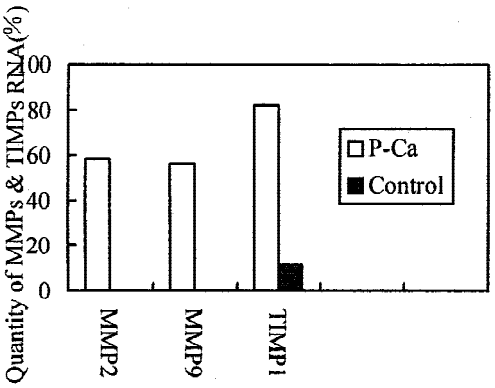

Fig. 1. Distribution of RNA signals of MMP and TIMP gene expression

ISH

Prehybridizations and hybridizations were carried out under stringent conditions in 50% formamide, 5 × Denhardt's, 10 m*M* DTT,10% dextran sulfate, 250 μg/ml sonicated human placental DNA, 250 μg/ml yeast t-RNA, 0.2% SDS, 0.75 *M* NaCl, 25 m*M* EDTA, and 25 m*M* PIPES, pH 6.8, at 50°C. After hybridization, slides were incubated with Rnase A at 37°C, followed by stringent washing procedures (0.25 × SSC at 50°C). After covering with photoemulsion (Huamei), all slides were exposed at 10°C for 2–4 weeks. Following development, sections were stained with hematoxylin and eosin and evaluated by standard light and darkfield microscopy using a COIC microscope (Chongqing, China). Staining results were semiquantitatively assessed without prior knowledge of any of the clinicopathological parameters. Both the intensity and distribution of ISH were considered in the definition of a positive sample. Staining was subjectively graded as weak (<30%), moderate (30%–80%), or intense (>80%), which depended on the percentage of positively stained tumor cells.

Statistical Analyses

Statistical comparisons were performed with the POMS software (Computer Resource Center, 4th Military Medical University, Xian, China), using the χ^2 test to determine the significance of the association between different variables. The level of significance was set at 0.05.

RESULTS

Increased levels of the mRNA of MMP2, MMP9, and TIMP1 genes [4], MMP2≈MMP9<TIMP1 (Fig. 1), were

TABLE I. Correlation of MMPs and TIMPs Gene Expression (Northern Blotting) Between Pancreatic Cancer Tissues and Pancreata of Organ Donors

	Pancreatic cancer tissues	Control	*P*
MMP2	58.4% ± 27.2%	0	
MMP9	56.5% ± 23.1%	0	<0.05
TIMP1	82.3% ± 39.7%	11.2% ± 6.9%	

TABLE II. Correlation of MMPs and TIMPs Gene Expression (ISH) Between Pancreatic Cancer Cells and Stromal Cells

	Pancreatic cancer cells	Stromal cells
MMP2	+	+~++
MMP9	+~++	+
TIMP1	+~++	+~++

found in two cell lines (PaTu8988, PaTu8902) as well as in pancreatic cancer tissues examined (Table I). Low levels of transcripts for MMP2, MMP9, and TIMP1 were detectable in pancreata of organ donors. Transcripts coding for MMP2, MMP9, and TIMP1 were found in both stroma and tumor cells [4]. However, MMP2 transcripts appeared to be more abundant in stromal cells, relatively more MMP9 transcripts were found in tumor cells, and TIMP1 transcripts were evenly distributed over tumor and stromal cells (Table II).

The 15 cases of pancreatic cancer were divided into two groups according to the diameter of tumor mass sections (Table III). In general, no significant correlation ($P > 0.05$) was shown between the two groups in gene expression of MMPs and TIMPs. The group of larger mass (diameter > 2 cm) shows a higher level of MMP2 gene expression than the group with a smaller mass (diameter $\leq$ 2 cm). A lower level of TIMP1 gene expression was observed in the group of larger mass (diameter > 2 cm) than that of the smaller group (diameter $\leq$ 2 cm).

Three groups were divided according to the detected level of serum CA19-9 (Table IV). More MMP gene expression was observed in the group of higher level of serum CA19-9. Obvious TIMP1 gene expression was shown in either group of higher or lower level of serum CA19-9. However, no significant correlation was found by statistical analysis ($P > 0.05$). Among the 15 cases, there were 12 cases of ductal carcinoma, with the highest percentage (80%), whereas acinar carcinoma had 6.7% and others 2.3% (Table V). A higher level of MMP and a lower level of TIMP1 gene expression were observed in the cases of ductal carcinoma with poor differentiation than in the cases with good differentiation. A significant correlation was shown ($P < 0.05$).

Among 15 cases of pancreatic cancer, there were 11 cases with abdominal lymphoid metastasis. MMP2 gene expression was detected in 5 cases of lymphoid infiltration, MM9 in 4, and TIMP1 in 7 (Table VI). MMP gene expression level was relatively higher in the lymphoid infiltration cases than in the lymphoid infiltration-free cases. Relatively lower levels of TIMP1 were found in the lymphoid infiltration cases than in those free of lymphoid infiltration ($P < 0.05$).

DISCUSSION

MMPs are a family of homologous Zn atom-dependent endopeptidases that are usually secreted from cells as inactive zymogens [5]. Collectively, these enzymes can degrade all of the components of the ECM, including fibrillar and nonfibrillar collagens, fibronectin, laminin, and basement membrane glycoproteins [6]. The expression of these enzymes is regulated at the transcriptional level by a variety of growth factors and oncogenes [7]. They are also regulated at the protein level by a family of specific inhibitors, the TIMPs [8]. MMPs and TIMPs are believed to have a role in the creation of the proteolytic defect in basement membrane type IV collagen [9]. In this study, distinguished levels were observed in the pancreatic cancer tissues for mRNAs of MMP, MMP9, and TIMP1 [4]. Transcripts coding for MMP2, MMP9, and TIMP1 were found in both stroma and tumor cells. Both stromal and tumor cells seem to be the sources of various members of MMPs and TIMPs in human pancreatic ductal adenocarcinoma. A majority of pancreatic cancer cells with overexpression of MMPs were localized to adencarcinoma nests and vascular lumens, indicating that these tumor cells have a strong potential to local invasion and distant metastasis.

Carcinoma of the pancreas usually produces symptoms when the tumor is far advanced (pain, weight loss, jaundice). Physical examination shows little. Chemotherapy and radiotherapy are of limited benefit. The prognosis is dismal. The molecular mechanism of local and distant metastasis is uncertain. MMPs may play a potent role in the course of metastasis of malignant tumors by a strong desmoplastic reaction. MMP gene expression correlated with the metastasis and poor prognosis in carcinoma of the intestine, stomach, breast, lung, head, and neck [1].

We showed that pancreatic cancer cells grew expansively (<2 cm) and capsulized with fibroblast cells. Direct infiltration was involved in paratumor tissues and capsules. However, those of tumor cells (>2 cm) showed visceral metastasis and lymphoid invasions [4]. In this study, gene expression of MMPs and TIMPs showed no correlation with the size of tumor mass (Table I). The results suggested that the gene expression did not contribute to the proliferation of the tumor cells. We might conclude that the tumor cells developed in a separate path in pancreatic ductal adenocarcinoma, different from that of metastasis. Although the size of the tumor mass is much smaller in the early stage, distant metastasis and postoperative recurrence might develop provided that the pathological classification is poor.

TABLE III. Correlations Among MMP2, MMP9, and TIMP1 Gene Expression and the Size of Tumor Mass

Tumor mass, diameter	N[a]	MMP2		MMP9		TIMP1	
		n[b]	%	*n*[b]	%	*n*[b]	%
≤2 cm	3	1	(1/3) 33.3	1	(1/3) 33.3	2	(2/3) 66.7
>2 cm	12	5	(5/12) 41.7	4	(4/12) 33.3	7	(7/12) 58.3
Total	15	6	(6/15) 40.0	5	(6/15) 40.0	9	(9/15) 60.0

[a]Number of samples in each group divided according to the diameter of tumor mass sections.
[b]Number of samples with gene expression of MMP2, MMP9, or TIMP1.

TABLE IV. Correlations Among MMP2, MMP9, and TIMP1 Gene Expression and the Level of Serum CA19-9

CA 19-9 (units/ml)	N[a]	MMP2		MMP9		TIMP1	
		n[b]	%	*n*[b]	%	*n*[b]	%
<31	3	1	(1/3) 33.3	1	(1/3) 33.3	1	(1/3) 33.3
31–150	5	2	(2/5) 40.0	1	(1/5) 20.0	3	(3/5) 60.0
>150	7	3	(3/7) 42.8	3	(3/7) 42.9	5	(5/7) 71.4
Total	15	6	(6/15) 40.0	5	(5/15) 33.3	9	(9/15) 60.0

[a]Number of samples in each group divided according to the level of serum CA19-9.
[b]Number of samples with gene expression of MMP2, MMP9, or TMP1.

TABLE V. Correlations Among MMP2, MMP9, and TIMP1 Gene Expression and the Pathological Classification

Pathological classification	N[a]	MMP2		MMP9		TIMP1	
		n[b]	%	*n*[b]	%	*n*[b]	%
Ductal carcinoma	12	4	(4/12) 33.3	3	(3/12) 33.3	6	(6/12) 50.0
Well differentiated	5	1	(1/5) 20.0	1	(1/5) 20.0	4	(4/5) 80.0
Poorly differentiated	7	3	(3/7) 42.9	2	(2/7) 28.6	2	(2/7) 28.6
Acinar carcinoma	1	1	(1/1) 100.0	1	(1/1) 100.0	1	(1/1) 100.0
Others	2	1	(1/2) 50.0	1	(1/2) 50.0	1	(1/2) 50.0
Total	15	6	(6/15) 40.0	5	(5/15) 33.3	9	(9/15) 60.0

[a]Number of samples in each group divided according to the pathological classification.
[b]Number of samples with gene expression of MMP2, MMP9, or TIMP1.

TABLE VI. Correlations Among MMP2, MMP2, and TIMP1 Gene Expression and the Lymphoid Infiltration

Lymphoid metastasis	N[a]	MMP2		MMP9		TIMP1	
		n[b]	%	*n*[b]	%	*n*[b]	%
Infiltration	11	5	(5/11) 45.4	4	(4/11) 36.4	7	(7/11) 81.9
No infiltration	4	1	(1/4) 25.0	1	(1/4) 22.2	2	(2/5) 50.0
Total	15	6	(6/15) 40.0	5	(5/15) 33.3	9	(9/16) 60.0

[a]Number of samples in each group divided according to the lymphoid infiltration.
[b]Number of samples with gene expression of MMP2, MMP9 or TIMP1.

CA19-9, the so-called gastroenterology-associated antigen, showed 90% accuracy in diagnosing pancreatic ductal adenocarcinoma [10]. However, gene expression of MMP2, MMP9, and TIMP1 has no correlation with the serum level of CA19-9 in this study, suggesting that CA19-9 is of no significance in the metastasis of pancreatic cancer.

Tumor cells of low differentiation without capsule infiltration progressed more easily in the early stage. Both capsule infiltration and lymphoid invasion are characteristic signs involved in local invasiveness and distant metastasis in pancreatic ductal adenocarcinoma. This study has shown that gene expression of MMP2, MMP9, and TIMP1 correlated with capsule infiltration and lymphoid invasion. MMP and TIMP gene expression also correlated with the pathological differentiation. It showed that

pancreatic cancer cells with a low degree of differentiation had a stronger potent for invasion and metastasis which increased as the disease progressed.

We concluded that an understanding of metalloproteinase expression and proteolytic activity might help physicians to assess the metastatic potential and prognosis of individual patients. MMPs and TIMPs are promising biological markers in the course of invasion and metastasis. The imbalance between MMPs and TIMPs may play a significant role in human pancreatic cancer.

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